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## Depression of mitochondrial metabolism by downregulation of cytoplasmic deacetylase, HDAC6

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### ABSTRACT

**Mitochondria perform multiple functions critical to the maintenance of cellular homeostasis. Here we report that the downregulation of histone deacetylase 6 (HDAC6) causes a reduction in the net activity of mitochondrial enzymes, including respiratory complex II and citrate synthase. HDAC6 deacetylase and ubiquitin-binding activities were both required for recovery of reduced mitochondrial metabolic activity due to the loss of HDAC6. Hsp90, a substrate of HDAC6, localizes to mitochondria and partly mediates the regulation of mitochondrial metabolic activity by HDAC6. Our finding suggests that HDAC6 regulates mitochondrial metabolism and might serve as a cellular homeostasis surveillance factor.**

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## 1. Introduction

Multiple biological functions of HDAC6 have been unveiled since the identification of histone deacetylase 6 (HDAC6) as a cytoplasmic deacetylase [1,2]. Early studies found that HDAC6-mediated  $\alpha$ -tubulin deacetylation destabilizes dynamic microtubules [3] and promotes an increase in cell motility [4,5]. In addition to  $\alpha$ -tubulin, several cytoplasmic proteins including Hsp90 [6,7], cortactin [8],  $\beta$ -catenin [9], peroxiredoxins I and II [10], and Ku70 [11] are regulated in a HDAC6-mediated deacetylation-dependent manner. Strong ubiquitin binding activity [12,13] further adds to the multifunctionality of HDAC6, enabling the regulation of many important processes including cell migration, cell stress response to the cytotoxic accumulation of protein aggregates, and immune synapse formation [1,2].

Here, we report the depression of mitochondrial metabolism by downregulation of HDAC6. Our findings uncover a novel function of HDAC6 as a homeostasis surveyor of the cell.

## 2. Materials and methods

### 2.1. Cell culture

All cells were maintained in DMEM supplemented with 10% heat-inactivated fetal calf serum. An established A549 cell line expressing RNAi for HDAC6 (HDAC6-KD), and HDAC6-KD cells re-introduced with wild type HDAC6 (KD + WT), catalytically inactive (KD + DC), or ubiquitin binding-deficient mutant (KD +  $\Delta$ UBD) have been described previously [14]. HDAC6-deficient mouse embryonic fibroblast (MEF) cell line is described in the literature [15].

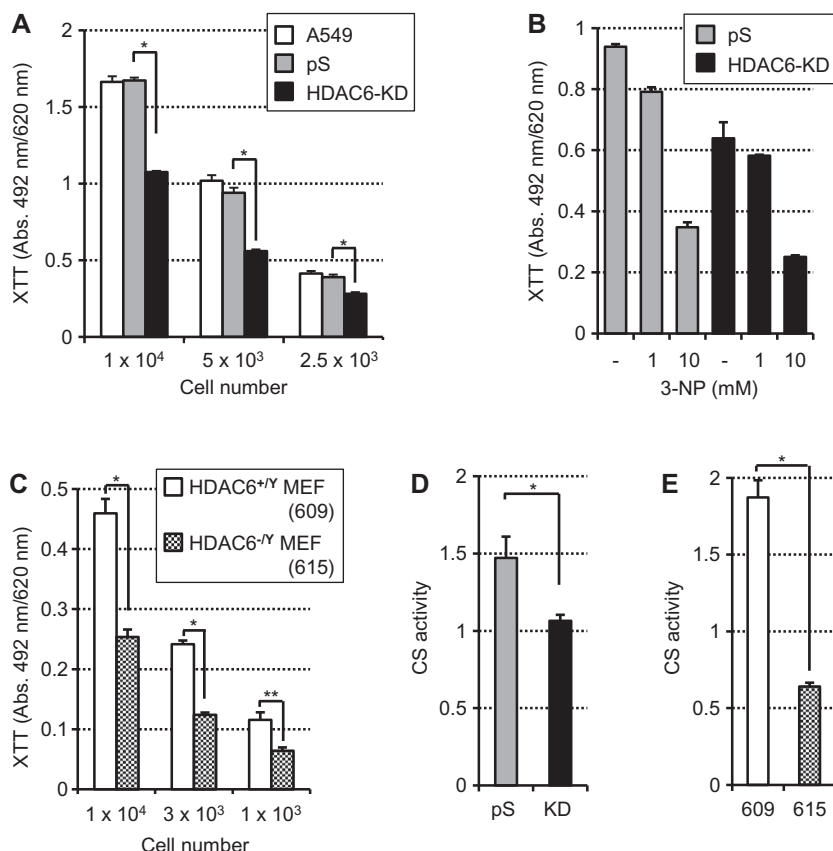
### 2.2. XTT assay

Cells were seeded in triplicate onto 96-well plates at the indicated density. The cleavage of XTT, sodium 3'-[1-(phenylamino)-carbonyl]-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzene-

**Abbreviations:** HDAC6, histone deacetylase 6; MEF, mouse embryonic fibroblast; CS, citrate synthase

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**Fig. 1.** Reduction of net mitochondrial metabolic activity in HDAC6-depleted cells. (A) XTT assay of HDAC6-depleted A549 cells. Data are presented as means  $\pm$  standard deviation (S.D.) from three independent assays.  $^*P < 0.01$ . HDAC6-KD, stable HDAC6-knockdown cells; pS, control cells for knockdown treatment. (B) XTT assay of HDAC6-depleted A549 cells treated for 6 h with 1 or 10 mM of 3-nitropropionic acid (3-NP). Data are presented as means  $\pm$  S.D. ( $n = 3$ ). (C) XTT assay of HDAC6-deficient mouse embryonic fibroblasts (MEFs). Data are presented as means  $\pm$  S.D. ( $n = 3$ ,  $^*P < 0.01$ ,  $^{**}P < 0.024$ ). 609, wild type MEFs; 615, HDAC6-deficient MEFs. (D) The citrate synthase (CS) activity of HDAC6-depleted A549 cells. Data are presented as means  $\pm$  S.D. ( $n = 3$ ,  $^*P < 0.03$ ). (E) The CS activity of HDAC6-deficient MEFs. Data are presented as means  $\pm$  S.D. ( $n = 3$ ,  $^*P < 0.01$ ).

sulfonic acid hydrate, by dehydrogenases in cells was measured after 8 h using Cell Proliferation Kit II (Roche). Plates were analyzed in a microtiter plate reader at 492 nm with a reference wavelength of 620 nm.

### 2.3. Citrate synthase (CS) assay

Cells were lysed ( $1 \times 10^6$  cells/200  $\mu$ l) in a buffer containing 0.1% Triton X-100, 1 mM EDTA, 50 mM Tris, pH 7.4, and Protease Inhibitor Cocktail (Nacalai Tesque). The CS assay was carried out using 20  $\mu$ l of the lysates in 96-well plates. CS activity was measured by adding 80  $\mu$ l of the reaction solution containing 0.1 mM DTNB, 0.3 mM acetyl-CoA, 1 mM oxaloacetate, and 50 mM Tris at pH 7.4 to each well. Absorbance was measured at 405 nm every 15 s for 10 min using the kinetic mode of a microtiter plate reader. Total protein concentration of the lysates was quantified by a Bio-Rad Protein Assay, and CS activity was normalized to the total protein concentration. CS activity was calculated as the rate of increase of absorbance with time.

### 2.4. Western blotting

Cells were lysed in SDS–polyacrylamide gel electrophoresis (PAGE) sample buffer. To detect proteins in Western blots, the samples were run on SDS–PAGE, electroblotted onto a polyvinylidene difluoride membrane (Millipore), and subjected to immunodetection using the appropriate primary antibody. Proteins were

visualized by using horseradish peroxidase-linked secondary antibody and enhanced chemiluminescence.

### 2.5. Subcellular fractionation and proteinase-sensitivity assay

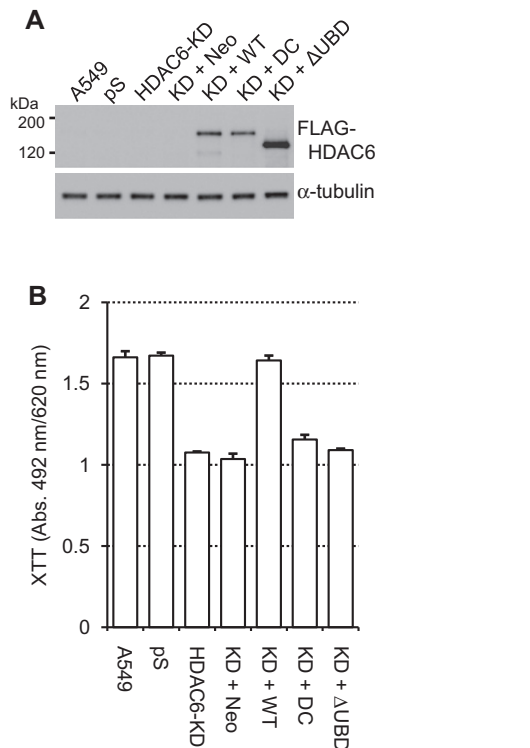
Cells ( $\sim 90\%$  confluence on a 15 cm dish) were collected and suspended in HEMS buffer consisting of 10 mM HEPES-KOH, pH 7.4, 1 mM EDTA, 220 mM mannitol, and 70 mM sucrose. Cells were then homogenized by a dounce homogenizer (7 ml) and conventional subcellular fractionation techniques were applied to homogenates as described previously [16].

The proteinase-sensitivity assay was performed as described previously [17], using HEMS buffer. Mitochondria (10  $\mu$ g) were treated with proteinase K at the indicated concentrations.

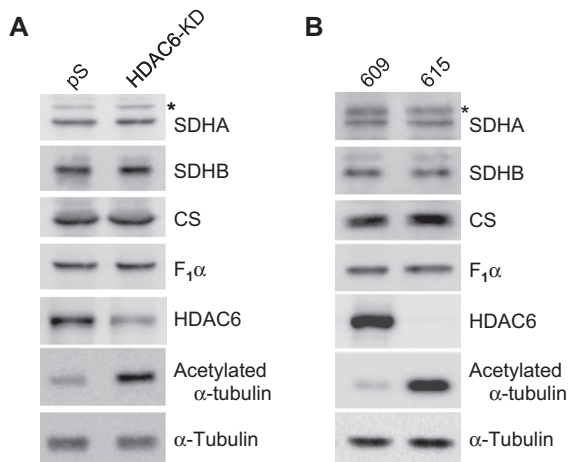
## 3. Results and discussion

### 3.1. HDAC6 expression correlates with mitochondrial metabolic activity

We have previously demonstrated that knockdown of HDAC6 expression causes a decrease in the steady-state levels of growth factor receptors [18]. During the study, we noticed that the down-regulation of HDAC6 influences mitochondrial metabolic activity. To confirm the effect of HDAC6 downregulation on mitochondrial metabolism, we first examined XTT assay-based dehydrogenase activity in HDAC6-KD cells. The net activity of dehydrogenases

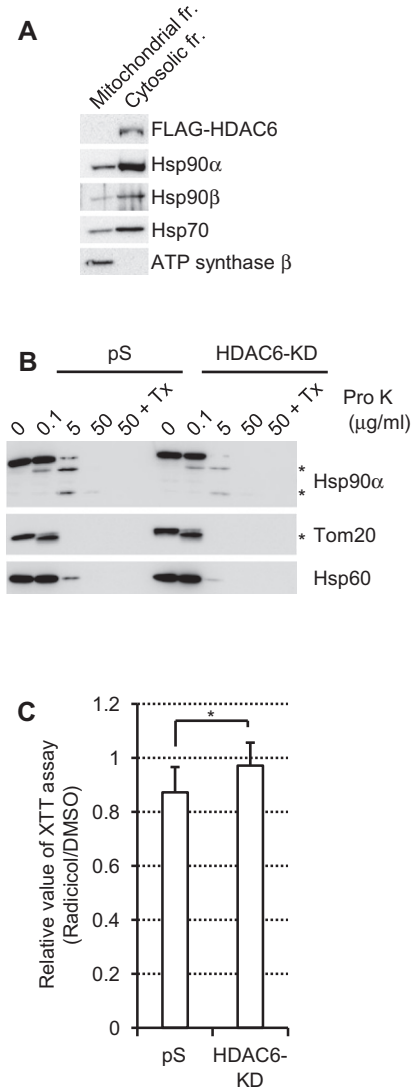


**Fig. 2.** Both deacetylase and ubiquitin binding activities of HDAC6 are linked to the regulation of mitochondrial metabolic activity. (A) Protein expression levels of re-introduced wild type and mutant HDAC6. Whole cell lysates were immunoblotted with anti-FLAG antibody.  $\alpha$ -Tubulin was used as loading control. The established cell lines used were HDAC6-KD cells re-introduced with wild type (KD + WT), catalytically inactive (KD + DC), and ubiquitin binding-deficient mutant (KD +  $\Delta$ UBD) of HDAC6. KD + Neo were control cells for re-introduction treatment. (B) XTT assay of HDAC6-KD cells re-introduced with HDAC6. Cells were seeded at a density of  $1 \times 10^4$  cells/well. Data are presented as means  $\pm$  S.D. ( $n = 3$ ).



**Fig. 3.** HDAC6 depletion does not affect the expression levels of nuclear-encoded mitochondrial enzymes. (A) Whole cell lysates from pS or HDAC6-KD cells were immunoblotted for the indicated proteins. (B) Whole cell lysates from HDAC6-deficient MEFs (615) or control MEFs (609) were immunoblotted for the indicated proteins. SDHA, complex II subunit 70 kDa Fp; SDHB, complex II subunit 30 kDa Ip;  $F_1\alpha$ , complex V ATP synthase subunit  $\alpha$  (as a mitochondrial control).  $\alpha$ -Tubulin was used as a loading control. An asterisk indicates a non-specific signal.

was significantly reduced in HDAC6-KD cells compared with control cells (A549 cells and pS cells; Fig. 1A). Succinate dehydrogenase is the major dehydrogenase responsible for formazan formation



**Fig. 4.** HDAC6 is distributed throughout the cytoplasm, and its substrate Hsp90 localizes in both the cytoplasm and mitochondria. (A) Both mitochondrial and cytosolic fractions were prepared from A549 cells stably expressing FLAG-tagged wild type HDAC6, and then immunoblotted with the indicated antibodies. Subcellular fractionation was confirmed by immunoblotting both fractions for Hsp70 (cytosolic protein) and ATP synthase  $\beta$  (mitochondrial protein). (B) Mitochondrial fractions from both HDAC6-KD and pS cells were subjected to a proteinase-sensitivity assay, and then immunoblotted with the indicated antibodies. Tom20 was used as a mitochondrial outer membrane protein, and Hsp60 as a mitochondrial matrix protein. Tx, 1% Triton X-100. Pro K, proteinase K. Asterisks indicate cleavage products. (C) The relative mitochondrial activity in radical-treated pS or HDAC6-KD cells. The mitochondrial metabolic activity of pS or HDAC6-KD cells treated for 24 h with DMSO or 100  $\mu$ M of radical was measured by the XTT assay. After correcting with cell numbers measured by calcein-AM staining, the relative mitochondrial activity was calculated by dividing the mitochondrial activity of radical-treated cells by the activity of DMSO control cells. Data are presented as means  $\pm$  S.D. ( $n = 5$ ,  $*P < 0.005$ ).

from XTT in mitochondrial respiratory complex II. In fact, 3-NP, an irreversible inhibitor of succinate dehydrogenase, reduced the XTT reaction in both pS and HDAC6-KD cells (Fig. 1B). In addition, HDAC6-deficient MEFs (615) also showed much lower dehydrogenase net activity than wild type MEFs (609) in the assay (Fig. 1C). We next examined mitochondrial oxidative capacity in HDAC6-KD cells using CS activity as a marker. CS activity in HDAC6-KD cells was reduced by 30% compared with that in pS cells (Fig. 1D). Compared with wild type MEFs, CS activity was also reduced by 60% in HDAC6-deficient MEFs (Fig. 1E). Consistent with our results, a gen-

ome-wide RNAi screening recently indicated that the knockdown of HDAC6 expression in *Drosophila* S2 cells correlated with a reduction in CS activity [19]. Thus HDAC6 expression correlates with mitochondrial metabolic activity in both insect and mammalian cells, and HDAC6 might have a role in the regulation of mitochondrial metabolic activity.

### 3.2. Both deacetylase and ubiquitin binding activities of HDAC6 are linked to the regulation of mitochondrial metabolic activity

HDAC6 is a cytoplasmic deacetylase which has double deacetylase domains and an ubiquitin binding domain [1,2,20]. To gain insight into the relevance of HDAC6 for mitochondrial metabolic activity, we tested the activities of HDAC6 to reveal which of these is required for the regulation of mitochondrial metabolic activity. We examined the net dehydrogenase activity in HDAC6-KD cells re-introduced with wild type (KD + WT), catalytically inactive (KD + DC), or ubiquitin binding-deficient mutants (KD +  $\Delta$ UBD) of HDAC6 (Fig. 2A). The reduced net activity of dehydrogenases in HDAC6-KD cells was fully recovered by the introduction of wild type HDAC6, but not by catalytically inactive or ubiquitin binding-deficient mutants (Fig. 2B). This result indicates that both deacetylase and ubiquitin binding activities of HDAC6 participate in the regulation of mitochondrial metabolic activity.

### 3.3. Loss of HDAC6 does not affect the expression levels of nuclear-encoded mitochondrial enzymes

Several lines of evidence have shown that HDAC6 affects the transcriptional activities of various factors despite its cytoplasmic localization [20]. The subunits of complex II and CS are nuclear-encoded mitochondrial enzymes; therefore, the reduction in their metabolic activities in HDAC6-depleted cells might be due to decreased gene expression in the absence of HDAC6. However, the expression levels of the complex II subunits, SDHA and SDHB, and CS were not affected by either knockdown of HDAC6 expression (Fig. 3A) or HDAC6 deficiency (Fig. 3B). In addition, both the mitochondrial mass and the apparent distribution of mitochondria in HDAC6-KD cells and HDAC6-deficient MEFs were indistinguishable from those of control cells (data not shown), indicating that mitochondrial biogenesis overall is unaffected by the loss of HDAC6.

### 3.4. HDAC6 regulates mitochondrial metabolic activity in the cytoplasm

A biochemical subcellular fractionation study showed that HDAC6 is distributed throughout the cytoplasm but not to the mitochondria (Fig. 4A). This indicates that HDAC6 may regulate mitochondrial metabolic activity from the cytoplasm. Of the known substrates of HDAC6, Hsp90 may be linked with the HDAC6-mediated regulation of mitochondrial metabolic activity. Inactivation of HDAC6 leads to hyperacetylation of Hsp90 and instability of its client proteins [6,7]. Interestingly, Kang et al. have found that an abundant pool of Hsp90 localizes to mitochondria in various tumor cells and regulates mitochondrial integrity [21]. We also found a considerable amount of Hsp90, especially Hsp90 $\alpha$ , in the mitochondrial fraction of both A549 cells (Fig. 4A) and MEFs (data not shown). Next we performed the proteinase K sensitivity assay using isolated mitochondrial fractions. Tom20, the mitochondrial outer membrane marker, was sensitive to proteinase K at a concentration of 0.1  $\mu$ g/ml, whereas Hsp60, the mitochondrial matrix marker, was insensitive at that concentration, and large portions were degraded at concentrations above 5  $\mu$ g/ml in both HDAC6-KD and control cells (Fig. 4B). Under these experimental conditions, most portions of Hsp90 $\alpha$  were insensitive to proteinase

K treatment at a concentration of 0.1  $\mu$ g/ml in both HDAC6-KD and control cells, which is reminiscent of the sensitivity of Hsp60 to proteinase K. These results indicate that Hsp90 $\alpha$  localizes to the inner space of the mitochondrial outer membrane, probably the matrix and intermembrane space, where most mitochondrial metabolic enzymes are located. This suggests that the reduction of mitochondrial metabolic activity from loss of HDAC6 is due in part to increased acetylation and inactivation of Hsp90. In fact, Hsp90 inhibitor radicicol reduced mitochondrial metabolic activity slightly, but significantly, in control cells but not in HDAC6-KD cells (Fig. 4C). Thus, we conclude that HDAC6 controls mitochondrial metabolic activity partly through deacetylation of Hsp90.

In addition to deacetylase activity, the ubiquitin binding activity of HDAC6 is simultaneously involved in the regulation of mitochondrial metabolic activity (Fig. 2). Mitochondrial protein quality control is regulated by the mitochondria-associated degradation pathway [22]. In this pathway, p97/VCP is recruited to stressed mitochondria and extracts ubiquitinated proteins from the outer mitochondrial membrane to the cytoplasm to present them to the proteasome for degradation [22]. Because p97/VCP is a known partner of HDAC6 [12] and regulates the fate of ubiquitinated cellular proteins together with HDAC6 [23], the reduced mitochondrial metabolic activity through loss of HDAC6 may be attributed to the stagnation of mitochondrial protein quality control. At present, we do not have any direct evidence demonstrating a link between deacetylase and ubiquitin binding activity of HDAC6 for the regulation of mitochondrial metabolic activity. We speculate, however, that together these two HDAC6 activities regulate mitochondrial metabolic activity because the lowered mitochondrial metabolic activity in HDAC6-KD cells fully recovered only by the introduction of wild type HDAC6 (Fig. 2).

Since mitochondria perform multiple functions critical to the maintenance of cellular homeostasis, the reduction of mitochondrial metabolism may impose a restriction of energy consumption on the cell. Indeed, loss of HDAC6 causes a reduction in cell motility both in NIH/3T3 fibroblasts [4,5] and lymphocytes [24]. HDAC6 may thus be a cellular homeostasis surveillance factor. It was proposed very recently that histone-modifying enzymes including kinases, acetyltransferases, methyltransferases, and O-GlcNAc transferase interpret the metabolic state of a given cell by changing chromatin modification patterns [25]. This proposal led us to the idea that HDAC6 could control the state of the epigenome through the control of mitochondrial metabolism. The functional relevance of HDAC6-mediated regulation of mitochondrial metabolism to epigenetics is an attractive issue yet to be unveiled.

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